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# **DEVELOPMENT OF BIOLOGICAL CONTROLS FOR NOXIOUS PLANT SPECIES**

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**General Outline of Work Proposed and Performed:** The proposed research on this contract consisted of the following objectives: 1) To discover and collect plant pathogens of selected weeds through field surveys in Florida; 2) determine the biological control ability of pathogens against their weed hosts in greenhouse and small-plot experiments; 3) confirm the host range and safety of pathogens that have bioherbicide potential in greenhouse trials; 4) develop methods to produce and formulate bioherbicides for field trials; and 5) evaluate the efficacy of the agents under field conditions. We have successfully met these objectives by a) discovering several pathogens of noxious weeds of importance to Florida and determining their biological control ability; b) identifying promising bioherbicide agents for cogongrass and other invasive grasses, tropical soda apple, melaleuca, Brazilian peppertree, and hydrilla; c) developing methods for large-scale inoculum production; d) establishing the efficacy and safety of some of the bioherbicide agents; and e) testing some of these agents in field trials. We are therefore on track to meet the ultimate goal of developing biological control agents to manage some of the noxious plant species in Florida, which will reduce the dependency on chemical herbicides for weed control and provide an environmentally beneficial weed management tool.

The project was funded for the period of 8/15/95 to 11/30/97. We received a one year No-Cost Extension until 11/30/98. The following is the final report of our work.

**Cogongrass (*Imperata cylindrica*):** We discovered and established the efficacy of two fungi as biological control agents for cogongrass. These fungi are undergoing testing in the field and the results are promising.

In 1996-97, infestations of cogongrass in and around Gainesville, Alachua county, FL were surveyed for diseased plants. Leaves of cogongrass exhibiting specks and lesions were used to isolate potential pathogens. Five isolates were obtained from diseased leaves collected from an infestation on Archer Road, Gainesville. Two of these five isolates were nonsporulating, two were *Curvularia* species, and one species was identified as *Bipolaris sacchari*. Collections outside Alachua County were also made. A sixth isolate (nonsporulating; identified as a *Rhizoctonia* sp.) was obtained from diseased plant material collected from Waldo, FL. Isolates from other grasses collected from south Florida were also considered as potential biocontrol agents. These isolates, *Drechslera gigantea*, *Exserohilum rostratum*, and *Exserohilum longirostratum*, were from large crabgrass (*Digitaria sanguinalis*), johnsongrass (*Sorghum halepense*), and crowfootgrass (*Dactyloctenium aegyptium*), respectively. Another isolate, a species of *Bipolaris*, was obtained from an *Echinochloa* sp. collected in Collier county, FL. An isolate of *Chaetomium fusiforme*, a pathogen previously reported on cogongrass (Chase et al., 1996; see publication list), was also included in the evaluation.

Greenhouse tests were conducted in 1997 to evaluate the pathogenicity of the 11 fungal isolates to cogongrass. Of the 11 fungal isolates, five caused mild leaf-spot reactions on the leaves while six others caused more severe reaction (leaf lesions and leaf blights). Those that caused lesions and blight were *Exserohilum rostratum*, *E. longirostratum*, *Drechslera gigantea*, and *Bipolaris sacchari* (Table 1). Two other isolates that caused leaf lesions and blighting were not considered for further evaluation since they did not sporulate readily and could not be identified. *Drechslera gigantea*, *E. longirostratum*, *E. rostratum*, and *B. sacchari* were studied further.

The biocontrol potential of all four pathogens was compared on the basis of the severity of disease they caused on cogongrass. Inoculations with the two *Exserohilum* isolates resulted in low disease severity (5-10%) while higher levels of disease severity (30-50%) were achieved with the application of the *D. gigantea* and *B. sacchari* isolates (all isolates were applied at the rate of  $10^4$ - $10^5$  spores/ml sprayed until run-off).

We developed methods to mass-produce spores for field trials. *D. gigantea* and *B. sacchari* readily produced spores on commonly used laboratory media, such as V8-juice agar and potato-dextrose agar (PDA), and natural media, such as grain seeds. The latter were particularly suitable for mass-production



of spores quickly and relatively cheaply. Sorghum, oat, and rye grains supported growth and abundant sporulation of the two fungi. Spores were produced within 7-14 days of incubation at 27° C, with 25 g of grains yielding 100 ml of spore suspension at 10<sup>5</sup>-10<sup>6</sup> spores/ml. A higher spore yield was obtained from cultures on rye grains (10<sup>6</sup> spores/ml). Spores from the grain cultures were viable and as infective as the spores harvested from V8 juice agar cultures. Thus, spores for greenhouse and mini-plot trials were mass-produced by inoculating sterilized rye grains with the fungal spore suspension and incubating at 27° C for 7-14 days (Fig. 1).

Table 1. Summary of Pathogenicity Tests of Fungal Isolates Screened Against Cogongrass

Isolate / Isolated from	Disease incidence <sup>a</sup>	Disease severity <sup>b</sup>
Untreated control	0	0
Fungus-free control	0	0
<i>Chaetomium fusiforme</i> (cogongrass)	18.5	13.0
<i>Curvularia</i> sp. (cogongrass)	36.5	11.0
<i>Bipolaris sacchari</i> (cogongrass)	95.3	93.0
<i>Bipolaris</i> sp. (cogongrass)	74.0	22.0
<i>Bipolaris</i> sp. (cogongrass)	66.6	11.0
<i>Drechslera gigantea</i> . (crabgrass)	94.4	35.0
<i>Exserohilum longirostratum</i> . (crowfootgrass)	81.5	17.5
<i>Exserohilum</i> sp. (johnsongrass)	57.1	6.0
<i>Bipolaris</i> sp. ( <i>Echinochloa</i> sp.)	0	0
<i>Bipolaris</i> sp. (cogongrass)	0	0
<i>Rhizoctonia</i> sp. (cogongrass)	0	0

<sup>a</sup> Percentage of leaves infected. <sup>b</sup> Percentage of the total foliage area diseased. Collectively, disease incidence and disease severity provide an estimate of the biological control ability of the pathogen.

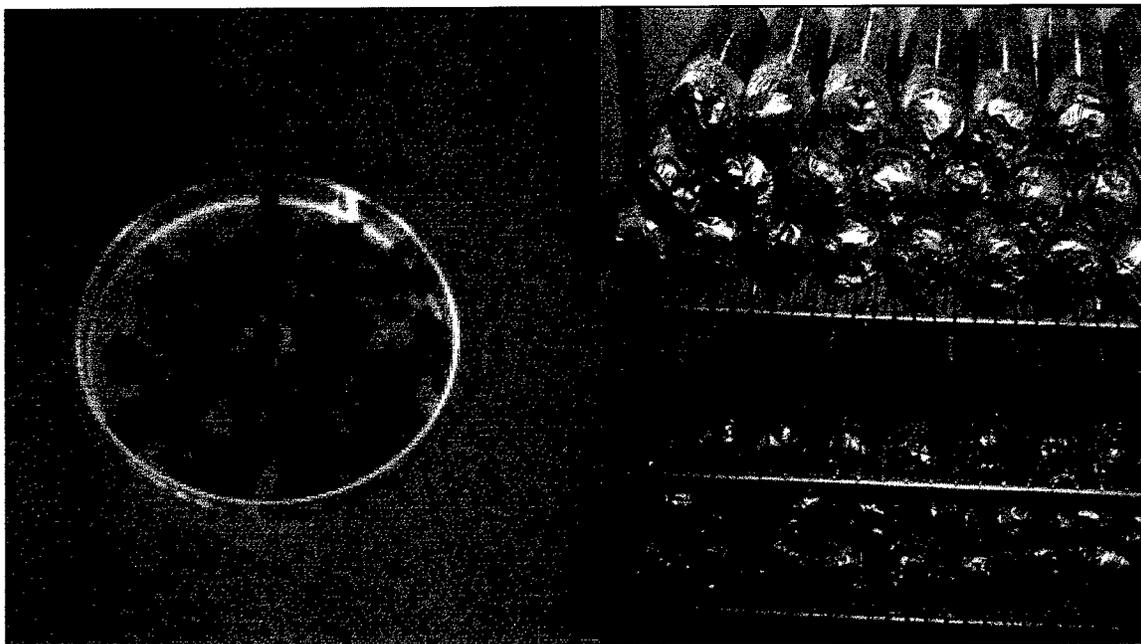
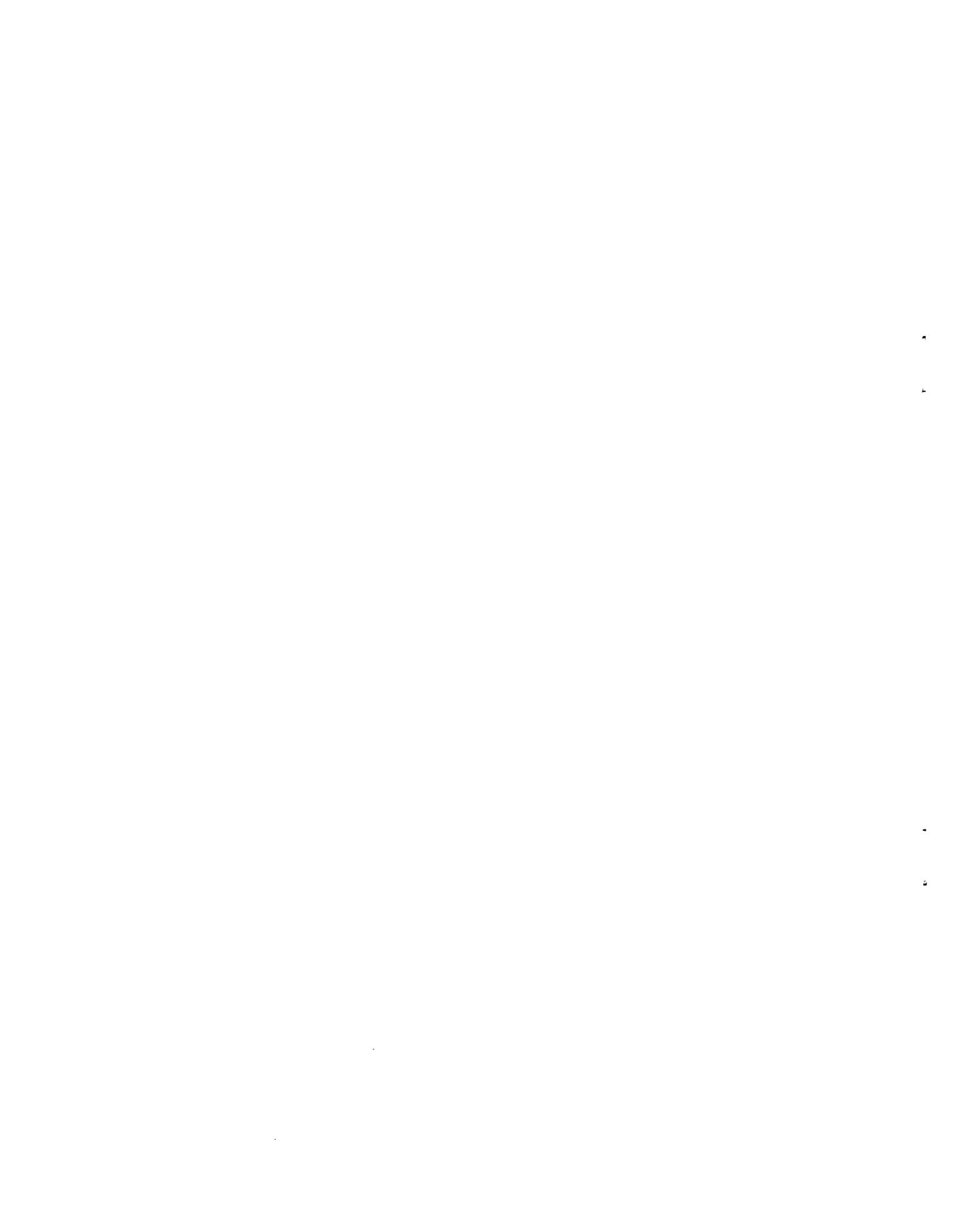


Figure 1. Sporulation of *B. sacchari* on rye grains (left). Mass-production of spores in bottled rye grains (right).

The effect of oil emulsions (4% and 40% Sunspray 6E® [SUNOCO] and mineral oil) and 1% gelatin solution were compared for their ability to enhance spore adhesion to the waxy leaf surfaces (Fig. 2). At 14



days after inoculation (DAI), 40% oil emulsion alone caused severe phytotoxic damage to cogongrass, resulting in 100% plant mortality. The application of *B. sacchari* spores in 4% oil emulsion resulted in 96.7% plant mortality within 14 DAI while 4% oil emulsion alone did not cause mortality until 21 DAI. At 21 DAI, phytotoxic damage and plant death were observed in plants treated with 4% oil emulsion alone but average mortality was 28.5%. Gelatin alone had no phytotoxic effect. The application of *B. sacchari* spores suspended in 1% gelatin solution resulted in 27.4% disease severity and 0% plant mortality.

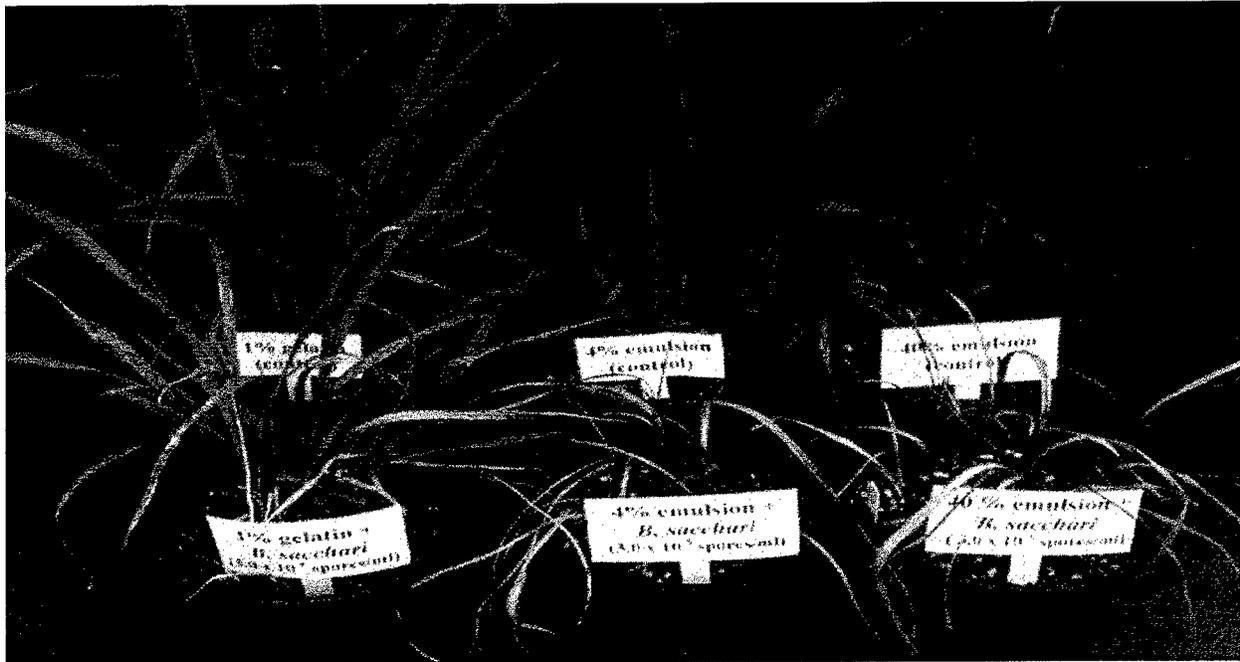


Figure 2. The effect of 1% gelatin, 4% oil emulsion, and 40% oil emulsion on the efficacy of *B. sacchari* as biocontrol agent of cogongrass. Back row (L-R): plants treated with 1% gelatin, 4% oil emulsion, and 40% oil emulsion alone. Front row (L-R): plants treated with 1% gelatin, 4% oil emulsion, and 40% oil emulsion, each containing *B. sacchari* spores.

The effect of dew-period length and adjuvants (gelatin and oil emulsion) on disease development and disease severity was also studied. *Bipolaris sacchari* spores suspended in 1% gelatin and 4% oil emulsion were applied to cogongrass plants (3-6 leaf stage; 5 weeks after sowing). Treated plants and controls were exposed to dew for 0, 4, 8, 12, and 24 h. Dew period and adjuvant type significantly affected disease ( $P=0.05$ ) (Fig. 3). Disease developed on all plants, regardless of the length of dew exposure. Disease severity was higher when 4% oil emulsion was used as adjuvant and when plants were exposed to greater than 4 h of dew.

The effect of initial temperature and adjuvant type (gelatin and oil emulsion) on disease development and severity was determined by exposing inoculated plants to different temperatures (21, 24, 27, 30, and 33 °C) and a 24-h dew period. Plants were then transferred to a greenhouse for further observation. Disease developed on all plants regardless of the initial temperature for 24 h. Adjuvant type had a significant effect on disease severity; disease severity was higher when oil emulsion was used as the adjuvant.

The optimum time of inoculation after clipping was also determined. Plants in pots were clipped at about 1.5 cm above the soil line and allowed to regrow for 1 to 5 weeks (to simulate mowing), and inoculated with spores of *B. sacchari* and *D. gigantea*, with 1% gelatin and 4% oil emulsion used as adjuvants. The results showed that age of plant regrowth and adjuvant type had significant effects on disease severity. Disease was more severe when 4% oil emulsion was used and when plants were inoculated at the early stage of regrowth.





Figure 3. The effect of dew-period length (0, 4, 8, 12, and 24 h) on cogongrass plants treated with *B. sacchari* spores in 1% gelatin (top) and 4% oil emulsion (bottom).



Figure 4. Cogongrass plants treated at 1, 2, 3, 4, and 5 weeks after clipping with spores of *D. gigantea* (left) and *B. sacchari* (right), with 4% oil emulsion used as the adjuvant.



A field trial was set up at Brooksville, FL using a completely randomized design consisting of replicated, 1-m<sup>2</sup> plots. Treatments were applied to natural infestations of mature cogongrass plants within the plots. The two isolates (*B. sacchari* and *D. gigantea*) were mixed with 4% oil emulsion and applied each separately and in combination. Appropriate carriers without the fungi were included. Disease development was observed 7 days after inoculation. Lesions and phytotoxic damage were seen on the leaves of the treated plants but the disease level was low. Diseased leaves that were plated to recover *B. sacchari* and *D. gigantea* from the respective treatments yielded the two isolates, confirming the pathogenicity of the isolates to cogongrass under field conditions.

Another field trial was set-up recently at the same location. In this study, the cogongrass was mowed two weeks prior to inoculation. In this trial we employed oil emulsions of different concentrations (8%, 16%, and 32%) to determine the best formulation that will increase the efficacy of the biocontrol agents. The results are pending.

**Other Invasive Grasses:** A novel method to control several weedy grasses was developed and successfully tested. The University of Florida has applied for a U.S. patent to protect the use of this technology and further development of this bioherbicide system appears quite feasible. Moreover, this strategy may be applicable for controlling cogongrass, as explained in the previous section, with a mixture of two pathogens, *B. sacchari* and *D. gigantea*.

In this method three or more host-specific fungal plant pathogens are combined and applied as a foliar spray to control several weeds simultaneously. In this approach, which we have termed the "Multiple-Pathogen Strategy", three fungi native to Florida, namely, *Drechslera gigantea*, *Exserohilum longirostratum*, and *Exserohilum rostratum* isolated from large crabgrass, crowfootgrass, and johnsongrass, respectively, were evaluated in greenhouse and field trials for their ability to control large crabgrass, crowfootgrass, guineagrass (*Panicum maximum*), johnsongrass, southern sandbur (*Cenchrus echinatus*), Texas panicum (*Panicum texanum*), and yellow foxtail (*Setaria glauca*). Grass seedlings of different ages (1-6 weeks after emergence [WAE]) were inoculated with spore suspensions of each pathogen containing 10<sup>5</sup> spores per ml. A mixture of the three pathogens (1:1:1 by vol) was also tested. The inoculum suspensions as well as the control (water only) were amended with 0.5% Metamucil®. The seedlings were sprayed to runoff, incubated in a dew chamber for 12 h at 28°C, and then held in a greenhouse. Disease incidence and disease severity ranged from 0-100% for the different grasses depending on plant age and inoculum concentration. At 10<sup>5</sup> spores per ml, younger seedlings (1 WAE) were more susceptible than older seedlings (2 WAE). At 2x10<sup>5</sup> spores per ml, even older seedlings (4WAE) were completely controlled. Further development of a bioherbicide to control grassy weeds based on the multiple-pathogen strategy appears feasible. As an example, the effect of *E. longirostratum* on eight different grasses is presented in Figure 5.

Safety of these pathogens to nontarget plants was established through host-range studies using individual pathogens (*D. gigantea*, *E. rostratum*, or *E. longirostratum*) as well as a mixture of all the three pathogens. Several economically important crop plants, including plants in the grass family, the Poaceae, were also screened. All crop plants tested were either immune or resistant to each of the three pathogens and the mixture of pathogens. The crop species tested (at 10<sup>5</sup> spores per ml), including corn (Asgrow), oats (FL502 and Fulghum), wheat (Triticale, Morey, and FL301), sorghum (TX398, DK104, and DK58), and rye (Greenacres and GrazeMaster) developed less than 1% disease when inoculated with *D. gigantea*. None of the crops developed disease when inoculated with *Exserohilum* spp. or the pathogen mixture. Thus, the results of the host-range study confirmed that *D. gigantea*, *E. rostratum*, and *E. longirostratum* and the mixture of all three pathogens were safe as bioherbicide agents.



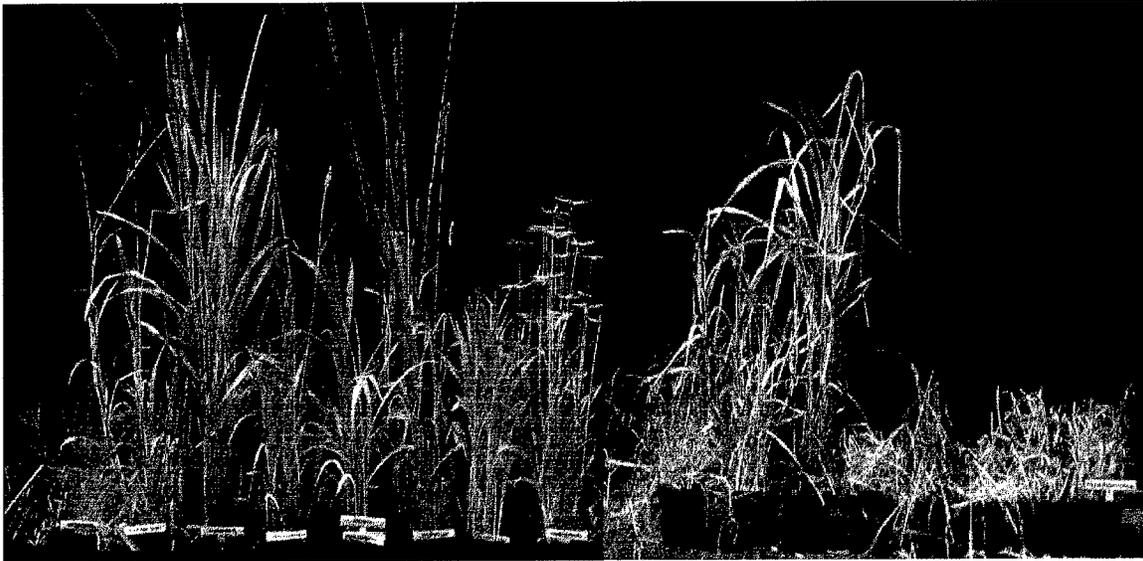


Figure 5. Effect of *Exserohilium longirostratum* from crowfootgrass on eight different grasses, from left to right in both pictures, crowfootgrass, Texas panicum, yellow foxtail, guineagrass, southern sandbur, johnsongrass, large crabgrass, and bermudagrass. Controls are in the left picture; plants inoculated with a foliar application of *E. longirostratum* spores are on the right. The pictures were taken two weeks after inoculation.

Field trials were conducted in 1996 and 1998 at Lake Alfred and Fort Pierce, FL, respectively. Treatments were laid out in a factorial randomized complete block design with fungal pathogen and carrier as the factors. The grass weeds were grown from seeds in a greenhouse and transplanted into each plot. Each of the pathogens ( $5 \times 10^5$  spores/ml) and a mixture of the three pathogens (1:1:1 v/v) were tested in three carriers: water, 0.05% Metamucil<sup>®</sup>, and a horticultural oil-based emulsion (Sunspray<sup>®</sup> 6E 80ml, paraffin oil 20 ml, spores in water 100 ml). The results indicated that it was possible to control several weedy grasses under field conditions using the three pathogens, and the control lasted for more than 3 months. An emulsion-based inoculum preparation of individual pathogens and a pathogen mixture controlled the following weeds: large crabgrass, crowfootgrass, johnsongrass, guineagrass, Texas panicum, southern sandbur, and yellow foxtail. Thus, *D. gigantea*, *E. longirostratum*, and *E. rostratum* have potential to be commercially developed as bioherbicides to manage weedy grasses.

The three fungi were also field-tested for their ability to control naturally established stands of guineagrass (Fig. 6). Testing was done in small plots (1 m<sup>2</sup>) arranged in a completely randomized factorial design and the experiment was done twice, in 1996 and 1998. The guineagrass plants within each plot were inoculated with spore suspensions of *D. gigantea*, *E. rostratum*, *E. longirostratum*, or a mixture of all three pathogens (1:1:1 by vol). The fungi were applied as foliar sprays, each containing  $5 \times 10^5$  spores per ml in water, 0.5% aqueous Metamucil<sup>®</sup>, or an emulsion (Sunspray 6E<sup>®</sup>). The carriers alone without the fungi were included as controls. A second application of treatments was done 2 weeks after the initial spray. Disease severity (DS) was recorded weekly for 4-6 weeks after the initial spray. In both trials, DS levels from the emulsion-inoculum treatments were higher than from the other two carrier-inoculum treatments. DS from the emulsion-inoculum treatments increased significantly after the second application and reached maximum 2 weeks later. The maximum DS levels from the emulsion-inoculum treatments of individual or mixture of pathogens were 93.8-98.5% (1996) and 96.1-98.0% (1998) compared to 1.5-7.25% (1996) and 9.0-18.5% (1998) from the water-inoculum, and 4.88-9.13% (1996) and 28.0-32.8% (1998) from the Metamucil-inoculum. Thus, effective control of guineagrass could be obtained under field conditions with an emulsion-based inoculum preparation.



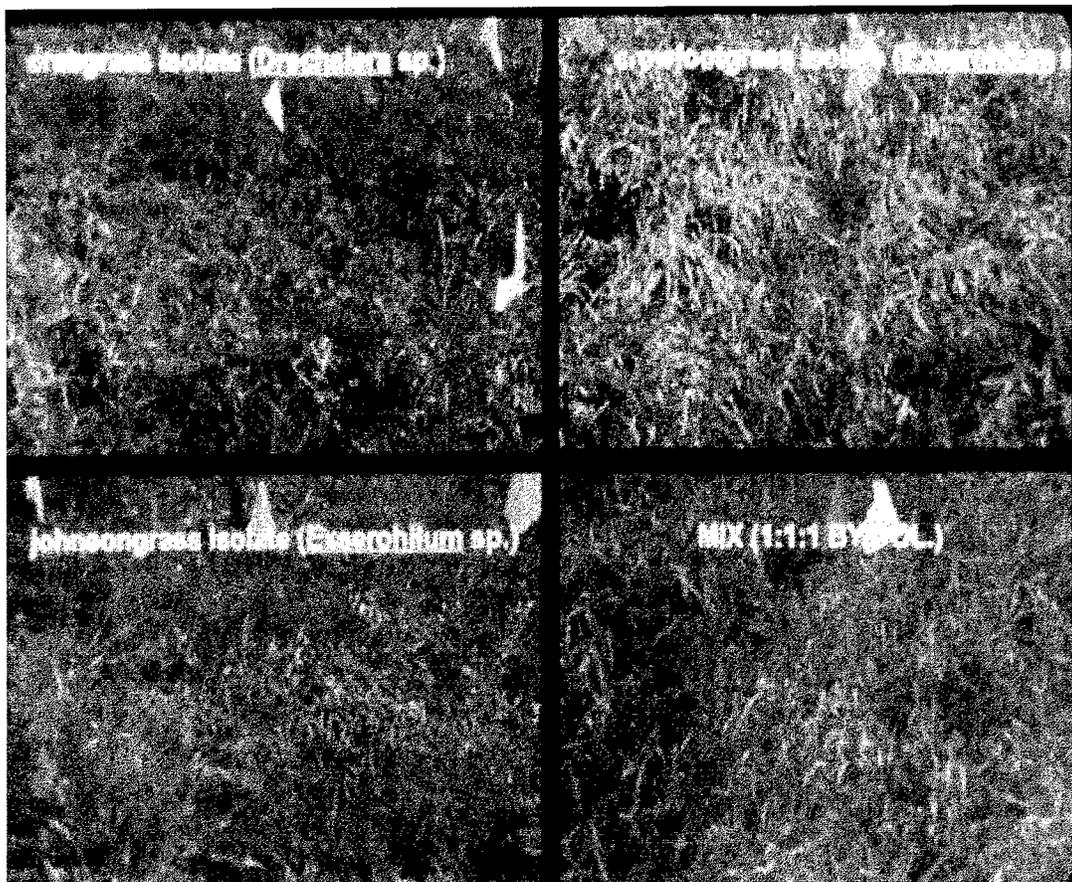


Figure 6. The effects of *D. gigantea*, *E. rostratum*, *E. longirostratum*, or a mixture of all three pathogens on guineagrass in a field trial.

To produce sufficient inoculum for field studies, a technique for mass-production and multiple-harvesting of *D. gigantea* and *E. rostratum*, based on a biphasic culturing system, was developed and tested. The steps involved in this technique are illustrated in Figure 7. The fungi were first grown from 1-week-old mycelial plugs in 1000 ml of V8 broth in 2-liter flasks. The inoculated flasks were shake-cultured (100 rpm) for 2-3 days at 25° C. The contents of each flask plus 10 ml of an antibiotic solution (3.7 mg/ml streptomycin and 2.5 mg/ml chloramphenicol) were blended in a Waring blender at a low speed for 30-60 sec and 500 ml of this suspension was poured onto a layer of V8 agar (500 ml) containing antibiotics (as above) in trays (37.5x30x1.25 cm) lined with aluminum foil. The trays were exposed to alternating light and dark cycles (12 h per cycle) at room temperature. The initial crop of spores appeared within 24 h. These spores were collected in two steps: first, the spores were gently scraped-off with a rubber spatula into sterile water. The remaining spores were then rinsed-off the agar surface with sterile water. The spore suspensions were pooled and the spores were allowed to settle down. The excess supernatant was decanted and the spores were resuspended in 250 ml sterile water. The trays were re-incubated under light as before and the spores were harvested twice at 24 h and 48 h. The spore yields for *D. gigantea* and *E. rostratum* averaged  $2.05 \times 10^5$ ,  $3.43 \times 10^5$ , and  $1.89 \times 10^5$ , and  $2.44 \times 10^5$ ,  $2.16 \times 10^5$ , and  $1.00 \times 10^5$  spores/ml/harvest, respectively. Thus, these fungi can be mass-produced by this biphasic culturing method and multiple spore harvests.





Figure 7. A technique for mass-production and multiple-harvesting of spores of *Drechslera gigantea* and *Exserohilum rostratum*. These pictures illustrate the steps involved in the biphasic culturing system described in this report.

**Tropical soda apple (*Solanum viarum*):** We made several field surveys to discover native pathogens that may be useful as biological control agents for tropical soda apple (TSA). Several pathogens were collected and tested in the greenhouse, and the most promising candidate, a bacterial pathogen, was also successfully tested in field trials. We are now in a position to seek permission to use this bacterium for commercial use in Florida.

The surveys yielded 45 fungal isolates belonging to species of *Alternaria*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Helminthosporium*, *Phomopsis*, and *Verticillium* that were isolated from diseased TSA plants. The isolates were tested for pathogenicity by spraying TSA plants with spores or mycelial fragments. Also



screened were five strains of *Ralstonia* (= *Pseudomonas*) *solanacearum*, the causal agent of the bacterial wilt of several solanaceous plants, and five strains of *Pseudomonas syringae* pathovar *tabaci*, the causal agent of the wildfire disease of tobacco. The bacteria were all from culture collections and they were tested by applying suspensions of cells in 0.25% Silvet L-77 (an organosilicone surfactant), as foliar sprays or by injecting water suspensions of cells directly into stems. The fungus-sprayed plants were incubated in a dew chamber for a minimum of 18 h before transferring to a greenhouse. The bacteria-sprayed plants were not subjected to the humidity-treatment and were inoculated and kept in the greenhouse. The experiments were replicated and included appropriate controls. None of the fungi produced any disease; therefore, all were regarded to be nonpathogenic to TSA. Strains of *P. syringae* pathovar *tabaci* were also nonpathogenic when sprayed or injected. The foliar application of *R. solanacearum* strains did not also produce any disease. However, two of the five *R. solanacearum* strains, both originally from tomato, were highly pathogenic when injected into TSA stem and induced a typical wilt disease characterized by massive foliar chlorosis and necrosis followed by plant death. The disease developed rapidly within four days after bacterial injection, and 100% of the inoculated seedlings were killed within a week. In a host-range trial using 31 *Solanum* spp., the *R. solanacearum* strains tested showed evidence of host-specialization. Thus, it may be possible to find strains that are specific to TSA, and we are exploring this possibility.

Concurrently, several additional strains of highly virulent *R. solanacearum* were obtained from culture collections and ranked for their ability to kill TSA. Since mowing is a recommended control practice for TSA, a post-mowing application of *R. solanacearum* is considered a rational method of field testing this bacterium. Initial trials were done on 187-day-old, 0.6-m-tall, containerized (11.4 liters) plants by clipping the main stem 3 cm above the soil and swabbing the cut surface with a 1-day-old bacterial suspension. The inoculum was applied at two rates, 1.74 and 0.74 absorbance units (AU) at 600 nm. A water control was included, and there were 5 replicates. By 12 weeks, 100% of the plants treated with the high inoculum level were killed (Fig. 8) and the shoot biomass was reduced in the low inoculum level treatment.

Two field trials were then established in Sumter and Levy counties, Florida, using a completely randomized design with 30 and 20 replicate plants per treatment, respectively. One-day-old inoculum was used at two rates: 1.04 AU (low rate, Sumter) and 1.96 AU (high rate, Levy). The Sumter site was in a pasture under full sunlight; the Levy site was under partial shade in an oak hammock. Treatments consisted of injecting the bacterial suspension into uncut stems of full-grown plants (inject treatment) or cutting the stems as described and swabbing the cut surface with the bacterial suspension. The results after 2 weeks for the Sumter and Levy sites are given in Table 2. *Ralstonia solanacearum* was more effective at the Levy site where the plants were shaded and smaller and higher inoculum level was used. Thus, *R. solanacearum* applied as a post-cut treatment is an effective way to control TSA under field conditions.

Table 2. Results of Field Trials with *R. solanacearum* as a Control for TSA.

Site	Treatment	Inoculum Rate	% Plants Wilted	% Failed to Sprout
Sumter County	Inject - Inoculation	Low	4	
	Inject - Control	Low	0	
	Swab - Inoculation	Low		78
	Swab - Control	Low		4
Levy County	Inject - Inoculation	High	45	
	Inject - Control	High	0	
	Swab - Inoculation	High		90
	Swab - Control	High		30

We now intend to apply to the Florida Department of Agriculture and Consumer Services and the U.S. EPA to obtain a permit to use *R. solanacearum* as a bioherbicide for TSA in Florida.



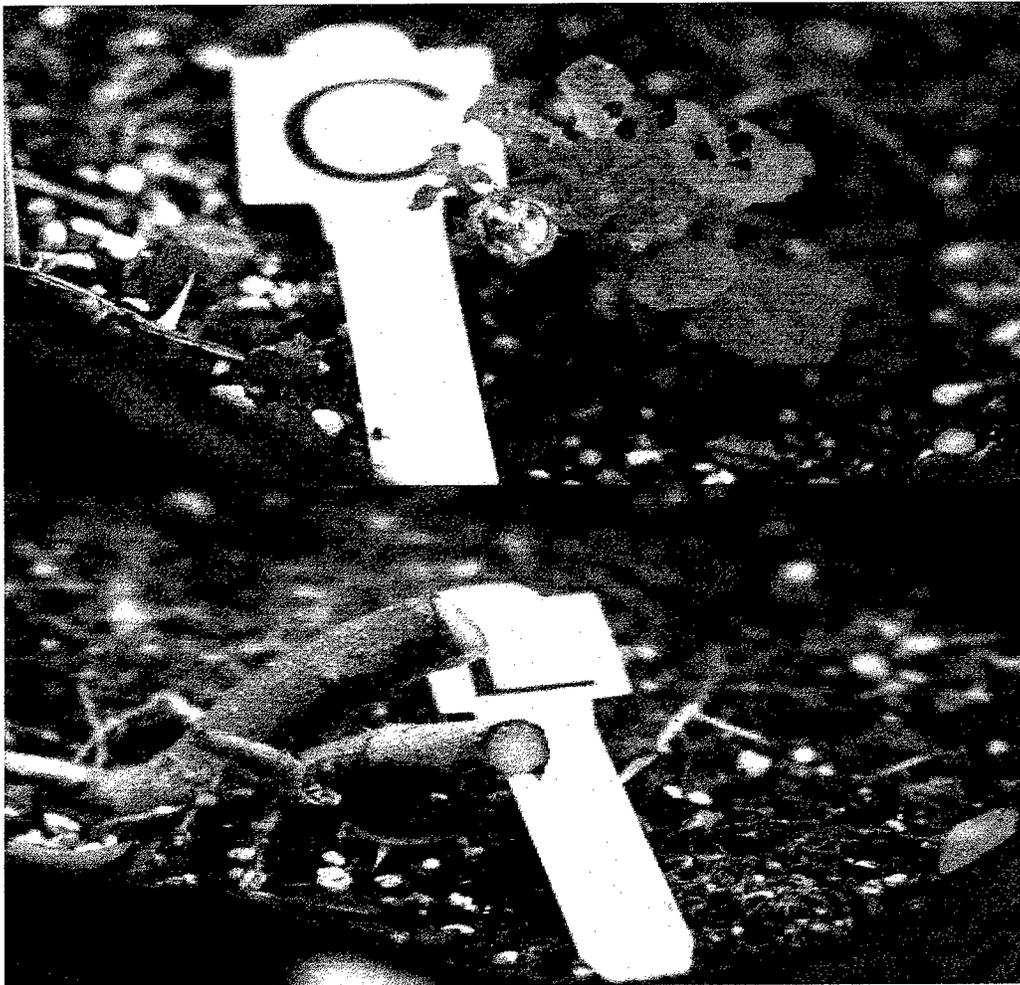
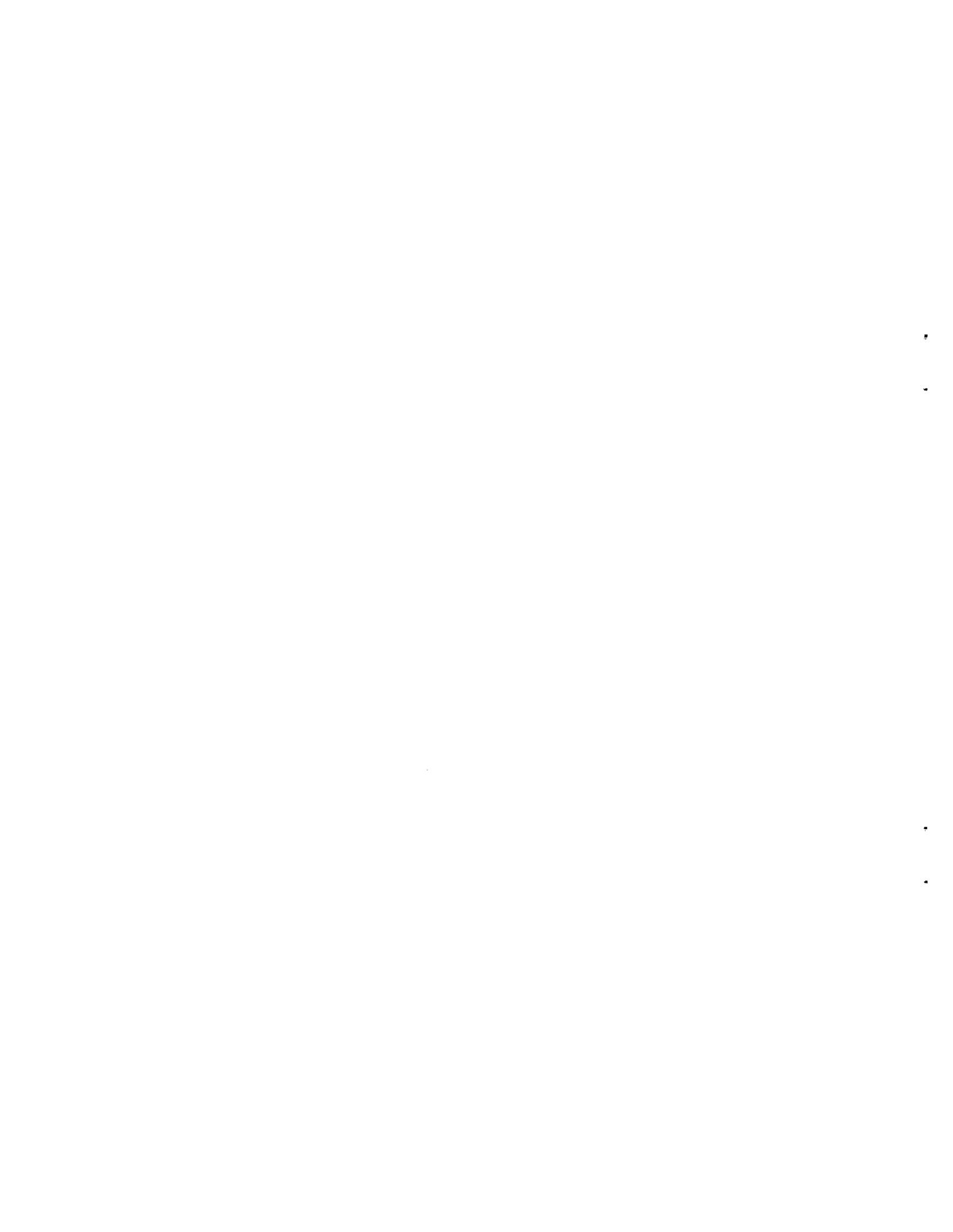


Figure 8. Biological control of tropical soda apple with *Ralstonia solanacearum*. The main stem of the plant was cut 3 cm above the soil and the cut surface was swabbed with water (control; top) or bacterial suspension (bottom). The inoculated cut plant is completely dead whereas the control plant has resprouted. Note the bacterial ooze on the cut surface of the inoculated plant.

***Melaleuca (Melaleuca quinquenervia)*:** We are evaluating two fungal pathogens as possible biological control agents of melaleuca: *Chondrostereum purpureum*, a wound-infecting basidiomycete that is naturally present in Florida and throughout North America, and a *Fusicoccum* sp. that we found to be naturally occurring on melaleuca in southwest Florida.

*Chondrostereum purpureum* is pathogenic to many broad-leaved tree species but not to conifers. It infects trees through wounds and can be used to kill cut tree stumps and prevent regrowth. It is currently being developed as a bioherbicide to control certain unwanted forest trees in the Netherlands and Canada. Since this fungus has a proven record of efficacy and safety, we obtained three isolates from the American Type Culture Collection, and, with permission from the Florida Department of Agriculture, initiated a field trial at a site in Ft. Myers. The isolates, ATCC 12283, ATCC 48890, and ATCC 60854, were grown on malt-extract-agar plates for 4 weeks and agar plugs containing mycelial growth were used as inoculum. Trees of various sizes and ages were inoculated with the isolates, 20 trees per isolate. Suitable control treatments were included. The trees were drilled (6 mm diameter by 4 cm deep into the wood tissue), five agar disks containing a fungal isolate were inserted into the drill hole, and the hole was covered with petroleum jelly.



The tree diameters at breast height and a map of the inoculated and control trees were kept, and the trees were examined for signs of disease. Six months after inoculation, five subsamples from each of the inoculated and control trees were harvested by cutting the trunks approximately 0.5 meters above and below the inoculation holes. The bark was removed and the trunks were split vertically along the middle to observe for the presence of dark coloration or staining, an indication of infection. If present, the length of the staining was measured and 20 pieces of wood were removed from the edges of the stain or at random from the cut trunk, surface-disinfested with 100% ethanol, and plated on potato-dextrose agar amended with 100 ppm penicillin. The plates were incubated at 30° C in the dark for 6 days and examined for *C. purpureum* colonies. Colonies were recovered and found to belong to *C. purpureum*. Thus, the results confirmed that *C. purpureum* is pathogenic to melaleuca.

During our surveys, we encountered a dieback disease of melaleuca trees in southwest Florida. The disease was characterized by yellowing (chlorosis) of the tops of infected trees, dieback of the tops, a gradual extension of the dieback symptoms towards the base of the trees, and noticeable wilting. Isolations from infected main stems and branches yielded a *Fusicoccum* sp. (teleomorph: *Botryosphaeria* sp.). On the basis of cultural, morphological, and pathological comparisons it was concluded that this isolate belonged to a new species of *Botryosphaeria* (anamorph: *Fusicoccum* sp.) that is different from *Botryosphaeria ribis*, previously implicated in a melaleuca stem canker and decline in southeast Florida (Rayachhetry et al., 1996). In greenhouse trials, the *Fusicoccum* sp. from southwest Florida was capable of rapidly killing melaleuca saplings (11 of 19 inoculated saplings [59%] killed in 17 days after inoculation).

A field trial was set up in Ft. Myers to test the effect of inoculation of melaleuca trees with three isolates of *Fusicoccum* sp. recovered from trees undergoing dieback. Melaleuca trees, 15 to 40 cm at dbh, were inoculated by drilling two 1-cm-diameter holes on the trunk at breast height and filling the holes with agar disks of *Fusicoccum* sp. isolates. An untreated control and a drilled control treated with plain agar block only were also included. The holes were covered with petroleum jelly to keep the agar disks moist. The trees were observed every 6 to 8 weeks for visible symptoms of dieback. An examination of cut trunks of trees inoculated in March 1997 confirmed that this fungus was capable of infecting melaleuca trees when injected into the trunk. Inoculated stem segments revealed dark, spreading discoloration of the wood, extending vertically in both directions from the drill holes. Such lesions were absent in the untreated control and drilled control treatments (Table 3; Fig. 9).

To prove that *Fusicoccum* is the causal agent of the trunk discoloration seen, 20 wood pieces were removed from the edges of the stain or at random from the cut trunk, surface-disinfested with 100% ethanol, and plated on potato-dextrose agar amended with 100 ppm penicillin. The plates were incubated at 30° C in the dark for 6 days and examined for *Fusicoccum* colonies. Eighty to 90% of the pieces yielded *Fusicoccum* isolates that were identical to the ones used for inoculation. Thus, *Fusicoccum* sp. was determined to be the causal agent of the observed dieback of melaleuca trees.

Host-range trials were conducted in a greenhouse to ascertain the potential of the *Fusicoccum* sp. from melaleuca to infect other nontarget tree species. The results indicated that saplings of *Eucalyptus grandis* and *Pinus elliotti*, when stem-inoculated with mycelial inoculum, were susceptible to this fungus whereas saplings of *Citrus* spp. were not. Seven other tree species common in Florida wetlands, namely bald cypress (*Taxodium distichum*), blackgum (*Nyssa sylvatica*), Brazilian peppertree (*Schinus terebinthifolius*), dahoon holly (*Ilex cassine*), pop ash (*Fraxinus caroliniana*), red maple (*Acer rubrum*), and sweet bay (*Magnolia virginiana*) were immune to this fungus. The finding of susceptibility of the eucalypt and pine seedlings is not surprising since *Botryosphaeria* spp. (which are the teleomorphs of *Fusicoccum* spp.) are common pathogens of many tree species.

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Figure 9. A dieback disease of melaleuca trees. *Fusicoccum* sp., a fungal pathogen was discovered as a causal agent of a dieback disease of melaleuca in southwest Florida. The dieback (upper left) appears to be spreading around the Ft. Myers area. The fungus is called *Fusicoccum* in its asexual (anamorphic) stage and *Botryosphaeria* sp. in the sexual (teleomorphic) stage. The fungus infects melaleuca when inoculated into the trunks (lower; the three trunks on the left were inoculated with the fungus and the two on the right are the controls). The fungus, when inoculated into the stems, kills melaleuca saplings (upper right; the three saplings on the right were inoculated with the fungus and the two on the left are controls).



Table 3. Length of Lesions on Trunks of Melaleuca Plants Inoculated with *Fusicoccum* sp. Isolates, Eight Months After Inoculation

<u>Treatment</u>	<u>Observation</u>	<u>Mean Lesion Length in cm</u>	
Untreated control	No lesions	0	a
Drilled, uninoculated control	No lesions; some discoloration present	1.02	b
Isolate M-8	Lesions present	20.1	d
Isolate M-9	Lesions present	7.9	c
Isolate M-10	Lesions present	12.7	c

Values are averages of five replicates. Means followed by the same letter are not significantly different ( $P=0.05$ ) using S-N-K multiple comparison test.

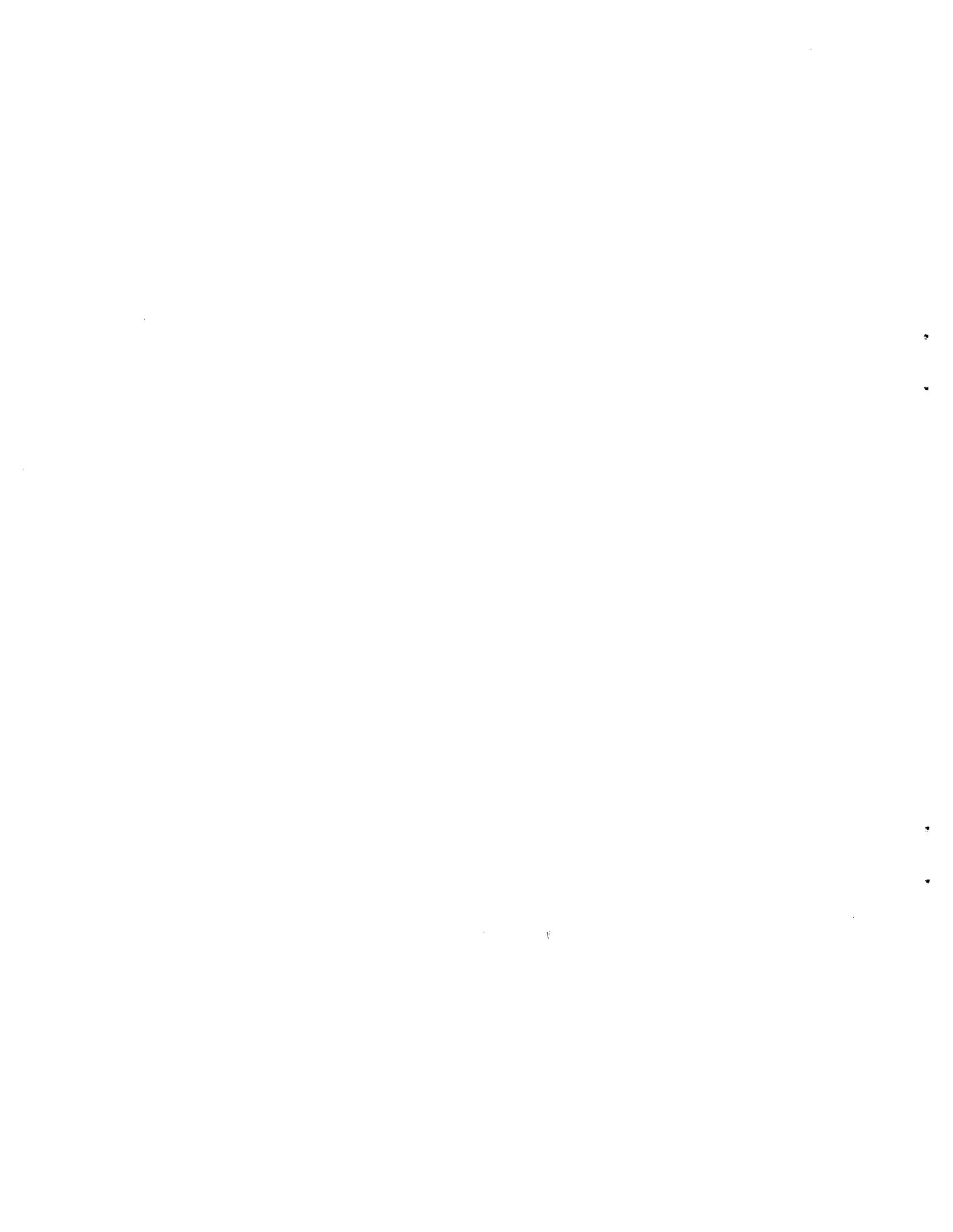
A field trial was initiated in south Florida (Highway 997, near Thompson Park) to confirm the ability of *Fusicoccum* sp. to cause dieback of regrowth from cut melaleuca stumps. The trial was set up in May 1997 and consisted of nine treatments, with 20 replicates (trees) per treatment. The trees were selected at random from an established stand of trees. The treatments were: 1) an untreated check; 2) a drilled, uninoculated check; 3) a cut, uninoculated check; 4) *Fusicoccum* sp. isolate Mel 8, applied as mycelial inoculum into drill-holes in the trunk; 5) same as # 4, but with isolate Mel 9; 6) same as # 4, but with isolate Mel 10; 7) cut stump, treated with wheat-bran formulation of Mel 8; 8) same as # 7, but with wheat-bran formulation of Mel 9; and 9) same as # 7, but with wheat-bran formulation of Mel 10.

For inoculation into drill holes, the *Fusicoccum* isolates (Mel 8, Mel 9, and Mel 10) were grown on potato-dextrose agar plates and four mycelial plug ( $4\text{ mm}^3$ ) were placed into a 5/16 inch diameter by 1 inch deep holes drilled into the trunks of trees and the holes were covered with petroleum jelly. For inoculation of cut stems with wheat-bran inoculum, the isolates were grown on autoclaved wheat-bran (2:1 bran:water, w/v) for 14 days at 25° C. The bran culture was suspended in sterile water (1:4 v/v) containing 1% xanthan gum (w/v) and 10% glycerol (w/v). This slurry was applied to the appropriate cut stem. Wheat-bran slurry similarly prepared without the fungus was used as a control. The plants were observed at 8 to 10 week intervals for signs of regrowth of stumps and dieback.

There was no sign of dieback in any of the drilled-inoculated treatments. However, there were signs of dieback in the cut treatments. In the latter group both fungus-treated and some control treatments had dieback of the regrowth. However, an analysis of variance of data from the most recent observations (19 months after inoculation) indicated no significant differences between treatments. Further observations will be made to see whether any delayed infectivity occurs. Additional studies are needed to evaluate fully the biocontrol potential of these *Fusicoccum* sp. isolates.

**Brazilian Peppertree (*Schinus terebinthifolius*):** The *Chondrostereum purpureum* isolates mentioned above and other fungal isolates collected from diseased Brazilian peppertree (BPT) plants in Florida and Brazil were evaluated. A stem tumor-inducing fungal pathogen, *Sphaeropsis tumefaciens*, appears promising. Work on this fungus is in a preliminary stage and further research is warranted.

The ability of *C. purpureum* to infect Brazilian BPT was evaluated under field conditions at a site in Hillsborough county. Similar to the study on melaleuca, BPT plants were inoculated through holes drilled in the trunk. The three isolates of *C. purpureum*, mentioned previously, were used. BPT plants were inoculated in August 1996 and examined at 6- to 8-week intervals to confirm pathogenicity of *C. purpureum* to this host. Five subsamples from each of the inoculated and control trees were harvested by cutting the trunks approximately 0.5 meters above and below the drill / inoculation holes where the inoculum disks or the agar blanks were placed. The bark was removed and the trunks were split vertically along the middle to observe for the presence of dark coloration or staining, an indication of infection. If any staining or coloration was found, the length of such markings (lesion) was measured. There was evidence of infection



of Brazilian peppertree by *C. purpureum* as (Table 4). To obtain proof of pathogenicity, 20 pieces of wood were removed from the edges of the stain or at random from the cut trunk, surface-disinfested with 100% ethanol, and plated on potato dextrose agar amended with 100 ppm penicillin. The plates were incubated at 30° C in the dark for 6 days and examined for *C. purpureum* colonies. The results confirmed that *C. purpureum* is pathogenic to BPT. However, the disease has not progressed to kill the plants. Therefore, we intend to evaluate alternative methods of application of the fungus, such as application to cut stems, to determine its potential usefulness.

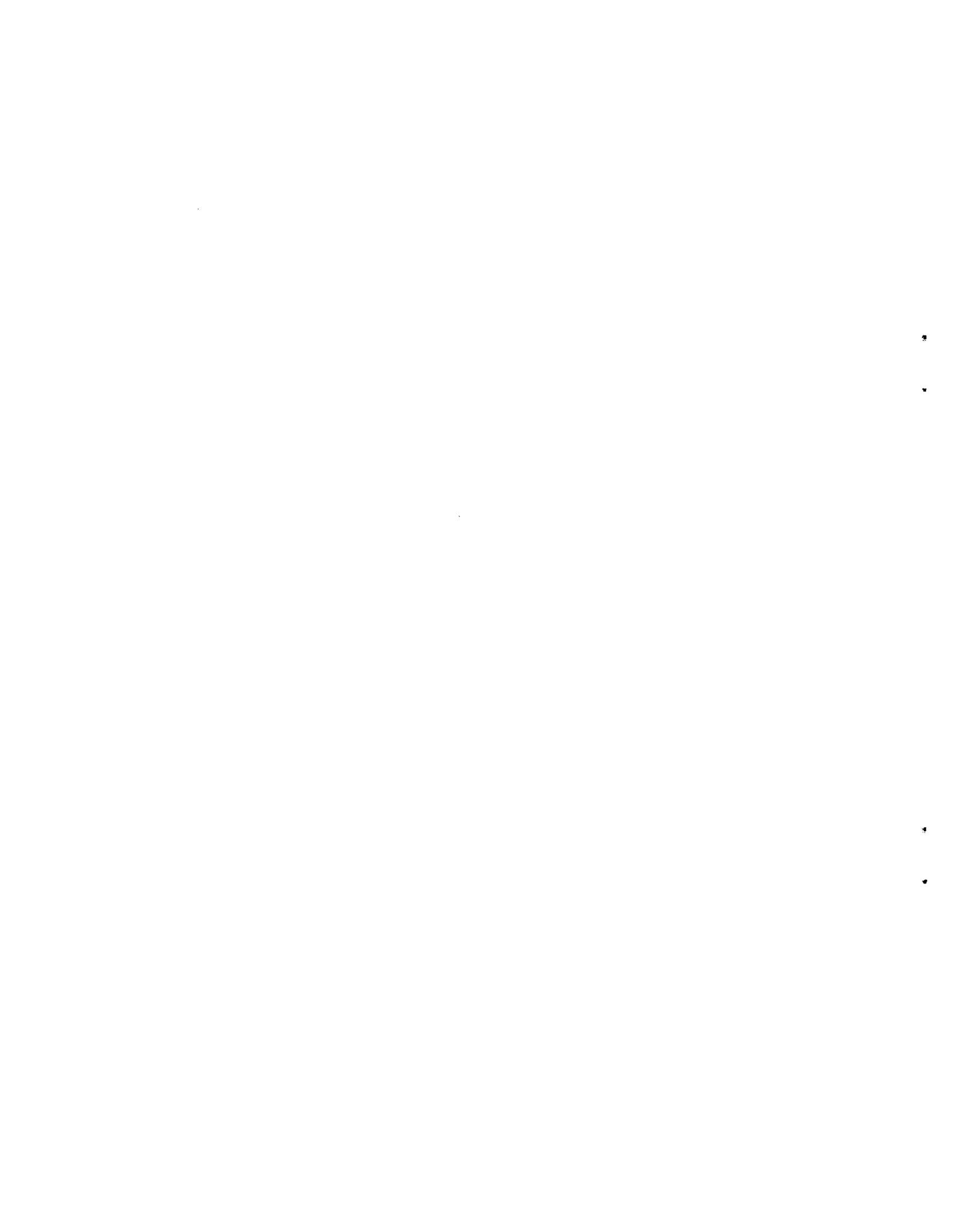
Table 4. Length of Lesions on Trunks of Brazilian Peppertree Plants Inoculated with *Chondrostereum purpureum* (CP) Isolates, Eight Months After Inoculation

Treatment	Mean Lesion Length in mm
Untreated control	0 d
Drilled, uninoculated control	0 d
Isolate CP ATCC12283	20.4 c
Isolate CP ATCC48890	28.7 b
Isolate CP ATCC00854	36.9 a

Values are averages of five replicates. Means followed by the same letter are not significantly different ( $P=0.05$ ) using S-N-K multiple comparison test.

Several BPT specimens suspected of having foliar diseases were collected from different locations in south Florida. Twenty-eight isolates were obtained from these specimens and tested for pathogenicity to BPT in a detached-leaf assay. Approximately 800 leaves from actively growing BPT plants were washed in Clorox:water:Tween 20 (1:10:0.1 by volume) for 30 seconds, washed twice in sterile water, and blotted dry before placing in a moist chamber made of petri plates. Each chamber held five replicate leaves which were treated with 0.5 ml of Tween 20 solution, injured with a 2-mm-diameter punch hole, and inoculated with a 3-mm<sup>2</sup> piece of agar containing the mycelium of the test isolate. Controls (leaves treated with agar pieces without fungal growth) were included. The plates were wrapped with Parafilm® and incubated at 24° C. The leaves were examined 3 and 5 days after inoculation, the lesions present were measured in two directions, and the data were analyzed. Seventeen of the 28 tested isolates produced leaf spots or lesions on detached leaves. Two isolates, 9c and 13d, caused significantly larger leaf lesions and 100% leaf necrosis after 7 days compared to the other isolates. Tentatively, these isolates have been identified to belong to *Rhizoctonia* sp. A second group of eight isolates produced a moderate level of lesion development and a third group of isolates induced only mild lesion development. Isolates 9c and 13d were evaluated further and determined to be the same fungus.

Isolate 9C was studied further. Disease symptoms caused by this isolate consisted of dark, reddish-purple necrotic lesions, with or without dry necrotic centers, and were distributed randomly over the leaf surface. To prove pathogenicity, the fungus was grown on PDA for 10-14 days, and the cultures blended with water in a food blender. Metamucil® was added to the fungal suspension at 0.5% w/v and the suspension was used to spray 2- and 3-month-old BPT seedlings. Seedlings were sprayed until the inoculum dripped off the foliage. Control seedlings were sprayed with water only, and the inoculated and control seedlings were maintained at 100% RH for 48 h and then moved to a greenhouse, and observed weekly. Clear symptoms of *Rhizoctonia* foliar blight were noticed after 10 days on the inoculated seedlings, and leaf lesions were observed on 94-100% of the inoculated seedlings in these separate trials. The symptoms produced in this experiment resembled the field symptoms that we recorded for this isolate. A *Rhizoctonia* sp. was reisolated from the lesions on the inoculated plants. A determination of the anastomosis group was performed by pairing the BPT isolate with "tester isolates," AG1-1A, AG2-2IV, AG-3, AG-4, and AG-5. In two separate tests, anastomosis (mycelial fusion indicative of compatibility of isolates) was observed between the recovered *Rhizoctonia* isolate and the tester strain AG2-2IV, confirming that the BPT isolate belonged to *Rhizoctonia solani*. This is the first report of *R. solani* causing a leaf-lesion disease



of BPT in Florida. Because this isolate caused only a mild level of disease damage on inoculated plants, it is not considered an effective biological control agent of BPT.

During our surveys we also observed a widespread stem-tumor disease of BPT in central and south Florida. This disease has been previously reported to occur on this host in Florida and the causal agent has been identified as *Sphaeropsis tumefaciens*. Samples of stem tumors were collected from several diseased BPT plants found in the vicinity of Vero Beach. Upon culturing from these tumors, 38 separate fungal cultures were obtained including many that were nonpathogenic. Two isolates (51597-2D and 51597-3B), considered likely to be pathogens, were evaluated further in greenhouse trials. Two known *Sphaeropsis* spp. isolates were included for comparisons. The host response was observed within 30 days of inoculation (Table 5). No erumpant stroma were observed with isolates 51597-2D and 51597-3B, but extensive stem and root necrosis was found. The infection (i.e., stem lesion and discoloration) extended from the initial point of inoculation to portions above and below this point. Diseased tissues were plated and the organisms growing from the tissues were recovered and found to be identical to the cultures used for inoculation. Pure cultures were prepared for further evaluation. Additional research is needed to determine the biocontrol potential of these isolates.

Table 5. Greenhouse Inoculation of 3-Month-Old *Schinus terebinthifolius* Seedlings with Fungi Isolated from BPT Stem Tumors.

<u>Treatments</u>	<u>No. Plants alive / No. tested</u>	<u>Mortality %</u>	<u>Tumor formation</u>
Untreated check	10/10 alive	0	No
Injured, nontreated check	10/10 alive	0	No
Injured, sealed check	10/10 alive	0	No
<i>Sphaeropsis</i> sp. from <i>Hypericum</i> sp.	8/10 alive	20	Yes
51597-2D	1/10 alive	90	No
51597-3B	1/10 alive	90	No
KJ-7 <i>Sphaeropsis sapinea</i>	10/10 alive	0	No

Four fungal cultures (38-1 through 38-4) recovered from BPT seedlings in Brazil were screened for pathogenicity to BPT in a quarantine greenhouse. Since these isolates were isolated from wilted BPT seedlings, they were considered likely to be root-infecting pathogens. Therefore, they were tested via root inoculation. However, the results indicated that these isolates are only mildly pathogenic and therefore not suitable as biological control agents of BPT.

**Hydrilla (*Hydrilla verticillata*):** During the past three years we isolated about 2200 microorganisms from hydrilla, surrounding water, and sediment collected from 10 man-made ponds and three natural lakes in Florida. A representative selection of fungi (272) and bacteria (78) from this collection were screened against hydrilla in a bioassay. The bioassay for fungi was conducted as follows: 2-week-old agar-plate cultures were flooded with 4 ml of sterile distilled water and the fungal growth was dislodged by thoroughly rubbing the agar surface. One ml of the resulting suspension was dispensed into 22-ml-diam x 150-ml-long glass tubes, each containing 49 ml of sterile tap water and a 9-cm-long healthy, terminal shoot of hydrilla. The hydrilla tubes were then covered with sterile glass beakers and placed under diurnal light (12 h light, 37  $\mu\text{E}/\text{m}^2/\text{s}$ ) at  $25 \pm 2^\circ \text{C}$  for 3 weeks. The bioassay for bacteria was conducted as follows: 3- to 4-day-old streaked culture plates (6-cm-diam) were flooded with 4 ml of sterile distilled water and the bacterial growth was dislodged as explained. One ml of the bacterial suspension was dispensed into a hydrilla tube and the same procedure as described was followed. Three replicates were used for each isolate. Three to 9 hydrilla tubes were left without microbial inoculation to serve as controls in each test. Three weeks after inoculation, the hydrilla was rated for severity of damage (DS). DS was determined for each shoot on a scale of 0 to 4, where 0 = healthy, 1 = 1-25% damage, 2 = 26-50% damage, 3 = 51-75% damage, and 4 = 76-100% damage (100% = complete kill). Of the 350 different isolates that were examined in this manner, none of the bacteria tested had significant effect on hydrilla, but 17 isolates of fungi (6.25% of the fungi

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tested; 4.86% of the total number of fungi and bacteria) were effective at DS level 4 on hydrilla 3 weeks after inoculation. Fungi that were capable of killing hydrilla are considered to have potential to serve as biocontrol agents for hydrilla or as sources of novel herbicidal metabolites. These include *Botrytis* sp., *Cephalosporium* spp., *Curvularia* sp., *Fusarium culmorum*, *Fusarium moniliforme*, *Phytophthora* sp., and unidentified species. They are undergoing further testing to characterize whether their activities are due to pathogenicity or phytotoxicity. Isolates that were most effective in killing hydrilla are ready to be tested in field trials using suitable inoculum formulations.

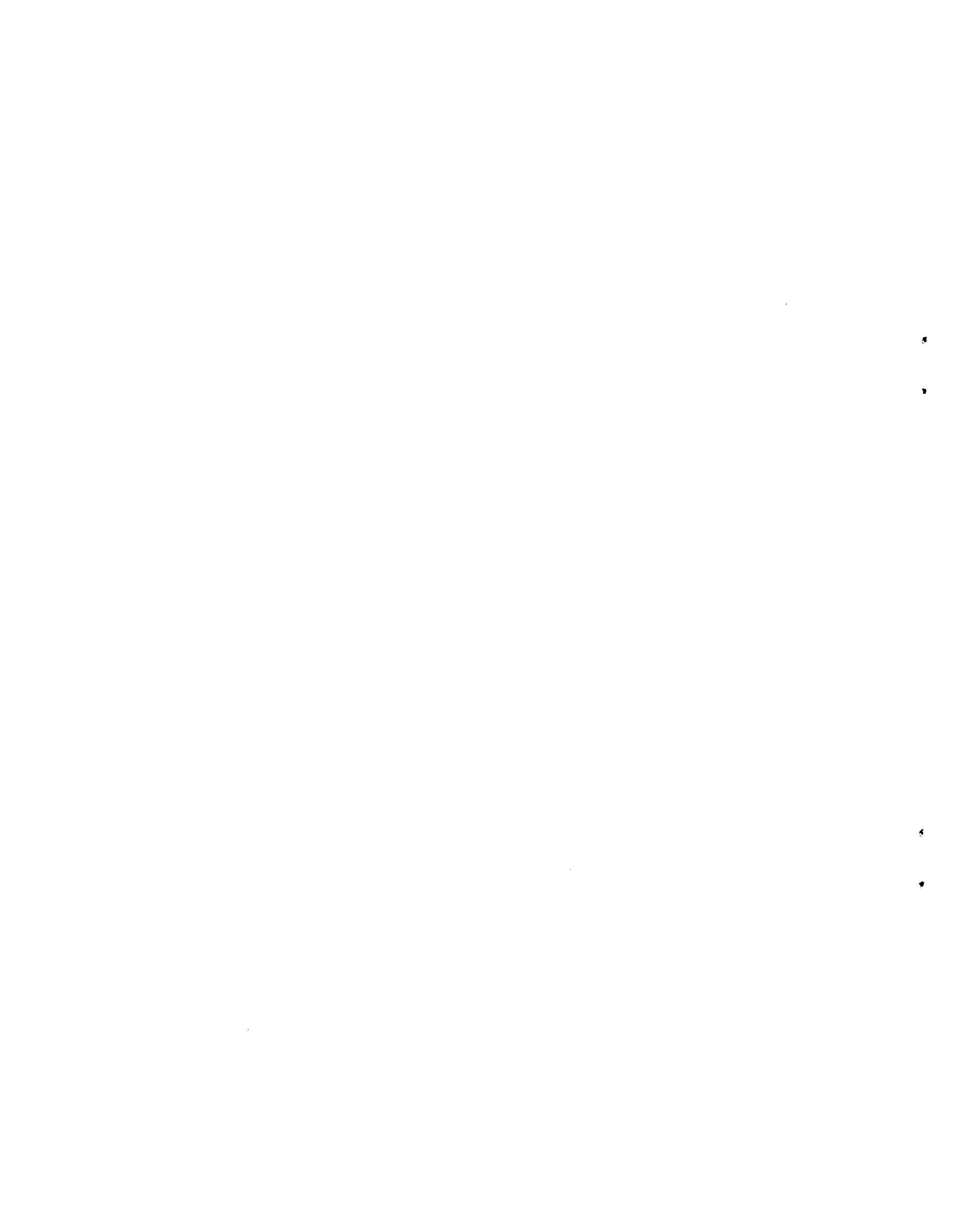
We have previously shown that an isolate of *Fusarium culmorum* is capable of controlling hydrilla in laboratory test systems. An epidemiological study was done to understand the effects of water quality, temperature, and inoculum level on disease development under field conditions. Initially three experiments were done to determine how spores (macroconidia) applied to water to control hydrilla settle in water and on hydrilla and how their viability is affected by water quality. The objective is to optimize inoculum retention and germination on hydrilla, and to maximize the efficacy of the applied conidial inoculum (see publications by Smither-Kopperl et al. under "Publications.").

Macroconidia were added to the surface of glass jars containing 275 ml of the following: distilled water, 5% Hoagland's solution + 0.1% KHCO<sub>3</sub>, Manatee Springs water, or Suwannee River water. Samples were taken with a Pasteur pipette at depths of 1, 4, 7.5, 11, 14, and 15 (bottom) cm at times 0, 0.5, 1, 1.5, 2, and 3 h after the conidia were added, and the distribution of the conidia was determined by microscopic observation. The spores were distributed at the top zones at time zero, but by 0.5 hours, they were distributed evenly throughout the liquid column. After 2 hours, the conidia were concentrated at the bottom layer. Thus it appears that the period of possible inoculum deposition will be limited by the rate of gravity-dependent settlement of the spores.

The viability of macroconidia over time was determined in the following sterile systems: deionized water, 5% Hoagland's solution + 0.1% KHCO<sub>3</sub>, 5% Hoagland's + 15% NaCl, 5% Hoagland's + 30% NaCl, Manatee Springs water, and Suwannee River water. Also, nonsterile waters from Manatee Springs and Suwannee River were used. Macroconidia were added at the rate of 1 ml containing 10<sup>6</sup> conidia per 200 ml of the above liquids in screw-cap bottles. One-ml samples were withdrawn after agitating the bottles at 0, 1, 2, 3, 4, 8, 12, 16, 20, 28, and 36 days. The number of colony forming units (cfu) was quantified by plating samples onto Komada's medium. After 1 week, the fungus could not be recovered from distilled water or Hoagland's + NaCl solutions. The fungus survived at the highest level in 5% Hoagland's and to a lesser extent in Suwannee River and Manatee Springs waters. The results indicated that water quality will significantly influence the survival of this fungus when used for controlling hydrilla. Further studies are needed to establish the exact duration of viability between times zero and 1 week, which is the critical period for host infection, penetration, and disease initiation. Results from these studies can then be used to design an effective bioherbicide agent and application methods.

**Talks at DOT Environmental Conference:** The Principal Investigator, R. Charudattan, presented two invited talks at the DOT Environmental Conferences held in Orlando on October 10, 1996 and in Palm Beach Gardens in September 23-24, 1998. A written paper titled "Biological Control of Noxious Weed Species using Plant Pathogens" and an abstract "Development of Biological Controls for Noxious Plant Species", were submitted to the agency.

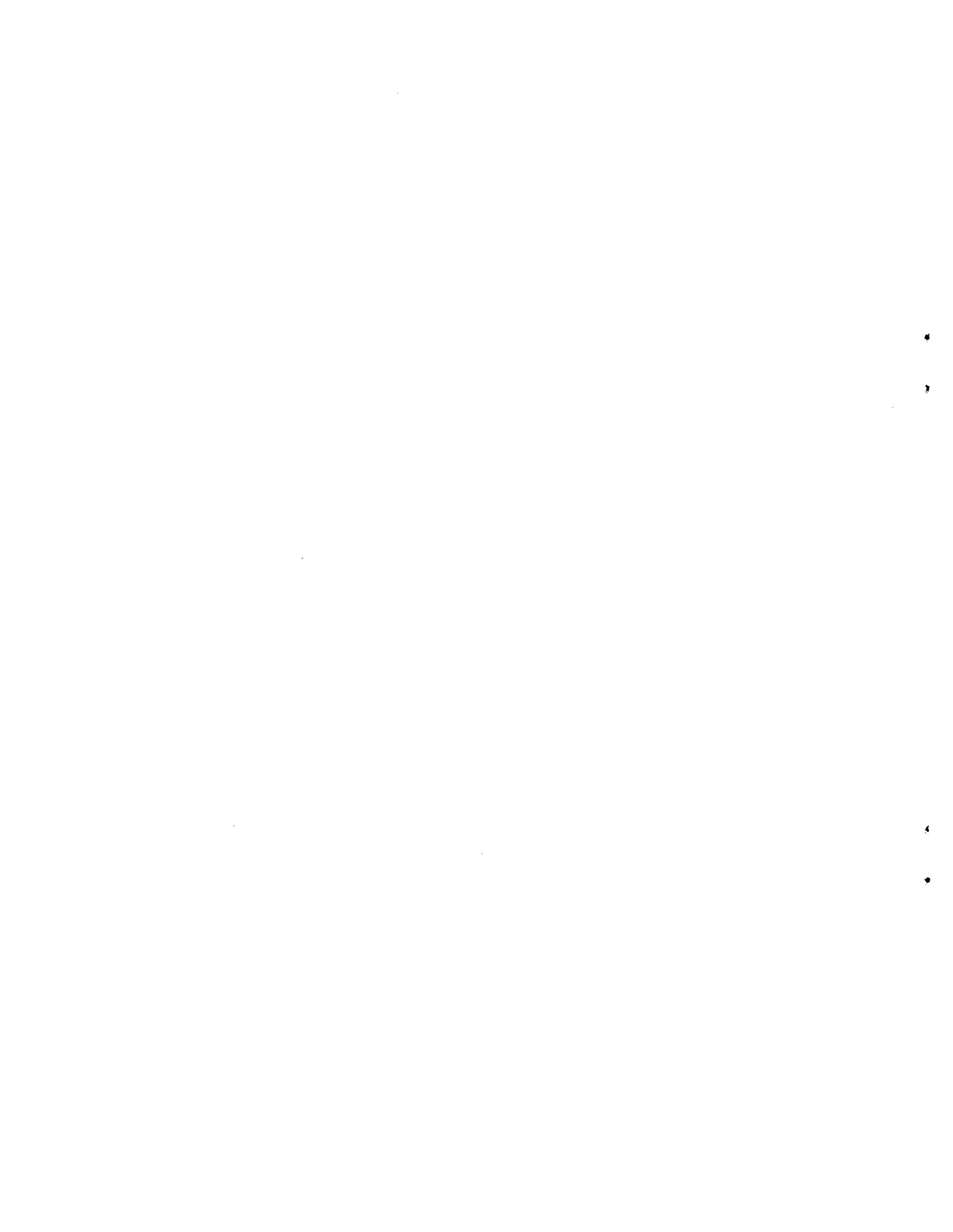
**Deliverables:** We have developed a feasible method of control for tropical soda apple by using the bacterial pathogen, *Ralstonia solanacearum*. We intend to seek clearances from the regulatory agencies (FLA DACS and EPA) to commercially use this bioherbicide agent to control soda apple in Florida. Two bioherbicide systems are under development for weedy grasses. The key element of our approach to controlling grasses is to use two or three pathogens in a multiple pathogen strategy. This approach has been successfully field-tested and a patent application has been submitted to the U.S. Patent Office to



protect this invention. We anticipate being able to control cogongrass and other invasive grasses with this strategy. We have also identified effective bioherbicide agents for hydrilla and these are now ready to be tested in field trials. Further work is needed to identify new pathogens and to develop some of the pathogens in our collection as biocontrol agents for Brazilian peppertree, melaleuca, and waterhyacinth. It is therefore highly important to continue this work to its successful completion in the next two years.

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